	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*		
Title: sigB PCR Protocol			
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sigB PCR **Protocol**

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to set forth standard guidelines for performing a PCR to amplify a 780 bp fragment of *sigB* to be used for sequence determination and subsequent phylogenetic analysis.

1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases, and the Milk Quality Improvement Program.

1.3 Definitions

Hot start PCR: Hot start PCR reduces non-specific amplification of DNA by inactivating the Taq polymerase at lower temperatures. It significantly reduces nonspecific priming, the formation of primer dimers, and often increases product yields. *Hot start PCR is required for sigB PCR, in contrast to most other PCR procedures.* This can be done either **manually** (by adding the taq polymerase, such as GoTaq, after the denaturation step), or **enzymatically** (using AmpliTaq Gold).
Master Mix: a mix of all the components required for PCR (taq polymerase if manual hot start, buffer, and $MgCl_2$), except the DNA template.
$MgCl_2$: a cofactor for the polymerase
AmpliTaq gold: a taq polymerase that activates at a higher temperature to prevent unspecific binding and annealing.
dNTPs: deoxyribonucleotide monomers, which are the building blocks of DNA. There are four dNTPs, cytosine, adenine, guanine and thymine
GoTaq Green: A PCR kit containing thermostable Taq polymerase, $MgCl_2$, and a PCR buffer which contains a green loading dye. This loading dye does not interfere with the PCR and saves the step of loading dye incorporation prior to running gel electrophoresis.

1.4 Safety

Be aware when handling BSL-2 pathogen. Appropriate protective measures need to be taken. All waste from these experiments needs to be treated as BSL-2 waste.

SECTION 2 MATERIALS

Single Tube Reactions

- Sterile water - Room 358B and Room 350A
- Sterile 0.2 mL tubes - Room 358B and Room 350A
- LM sigB15 (forward) Primer, 12.5µM (5' - AAT ATA TTA ATG AAA AGC AGG TGG AG -3') - Room 352C, chest freezer
- LM sigB16 (reverse) Primer, 12.5µM (5' - ATA AAT TAT TTG ATT CAA CTG CCT T -3') - Room 352C, chest freezer
- GoTaq Flexi Polymerase - Room 352, "Dumb" freezer
    - ✦ OR: AmpliTaq gold DNA Polymerase - Room 352, "Dumb" freezer
- GoTaq Green 5x PCR Buffer - Room 352, "Dumb" freezer
    - ✦ OR: AmpliTaq 10X PCR buffer - Room 352, "Dumb" freezer
- GoTaq $MgCl_2$, 25 mM - Room 352, "Dumb" freezer
    - ✦ OR: AmpliTaq $MgCl_2$ (25mM) - Room 352, "Dumb" freezer
- dNTP solution consisting of 1 mM each dATP, dGTP, dCTP, dTTP dNTP stocks in Room 352, "Dumber" freezer, in covered cryoblock
- Vortex Mixer - Room 358B
- Miniature Centrifuge - Room 358B
- Thermocycler - Room 356
- Micropipette and sterile filter tips
- Crushed Ice

High Throughput Reactions (96-well plate)

- Sterile reservoir basin - Room 358B
- Sterile 96-well plates - Room 358B
- Multi-channel pipette
- Sterile water - Room 358B and Room 350A
- LM sigB15 (forward) Primer, 12.5µM (5' - AAT ATA TTA ATG AAA AGC AGG TGG AG -3') - Room 352C, chest freezer
- LM sigB16 (reverse) Primer, 12.5µM (5' - ATA AAT TAT TTG ATT CAA CTG CCT T -3') - Room 352C, chest freezer
- GoTaq Flexi Polymerase - Room 352, "Dumb" freezer
    - ✦ OR: AmpliTaq gold DNA Polymerase - Room 352, "Dumb" freezer
- GoTaq Green 5x PCR Buffer - Room 352, "Dumb" freezer
    - ✦ OR: AmpliTaq 10X PCR buffer - Room 352, "Dumb" freezer
- GoTaq $MgCl_2$, 25 mM - Room 352, "Dumb" freezer
    - ✦ OR: AmpliTaq $MgCl_2$ (25mM) - Room 352, "Dumb" freezer
- dNTP solution consisting of 1 mM each dATP, dGTP, dCTP, dTTP dNTP stocks in Room 352, "Dumber" freezer, in covered cryoblock
- Vortex Mixer - Room 358B
- Miniature Centrifuge - Room 358B
- Thermocycler - Room 356
- Adhesive aluminum foil lids - Room 358B
- Micropipette and sterile filter tips
- Crushed Ice

SECTION 3 PROCEDURES

3.1 Lysate Preparation

Refer to the "Preparing DNA Lysates" SOP on the Food Safety Lab wiki.

3.2 PCR Amplification

3.2.1 Working in room 358B, prepare a master mix of the components listed in Table 1 by pipetting each component into a sterile 1.5 mL microcentrifuge tube (and a 0.2 mL PCR tube for the Taq Mix if using GoTaq).

3.2.1.1 Pipette up and down to mix after each addition and to ensure that no reagent clings to the pipette tip.

3.2.1.2 Store all master mix components and the prepared master mix on crushed ice.

3.2.1.3 Vortex and centrifuge down the contents of all reagent containers before opening (DO NOT vortex the Taq or any solutions containing Taq, flick the tube to mix these solutions)

3.2.1.4 Calculate master mix volumes required to include positive and negative controls and 1-2 additional reactions to compensate for pipetting errors.

Table 1: sigB PCR Master Mix

Manual Hot Start (GoTaq):

Master Mix:	1X (50µL rxn)
• dH_20	20.5 µL
• 5X PCR buffer*	8.0 µL
• $MgCl_2$ (25mM)	3.0 µL
• dNTPs (1mM)	2.5 µL
• SigB15 (12.5µM)	2.0 µL
• SigB16 (12.5µM)	2.0 µL
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	38 µL per tube

Taq Mix:	1X (50µL rxn)
• dH_20	7.6 µL
• 5X PCR buffer*	2.0 µL
• Taq polymerase (GoTaq)	0.4 µL
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	10 µL per tube

Enzymatic Hot Start (AmpliTaq):

Master Mix:	1X (50µL rxn)
• dH_20	32.75 µL
• AmpliTaq 10X PCR buffer	5.0 µL
• AmpliTaq $MgCl_2$ (25mM)	4.0 µL
• dNTPs (1mM)	2.0 µL
• LM sigB15 primer (12.5µM)	2.0 µL
• LM sigB16 primer (12.5µM)	2.0 µL
• AmpliTaq gold polymerase	0.25 µL
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	48 µL per tube

*adjust volumes if buffer is 10X instead of 5X

3.2.3 Manual Hot Start (GoTaq Polymerase)

3.2.3.1 Dispense 38 µL of prepared master mix (but not the Taq Mix) into one well per lysate in a 96-well PCR plate or into individual 0.2 mL PCR tubes. Cover the plate loosely or cover the tubes and hold on ice.

3.2.3.2 Remove the plate or tubes containing the master mix from room 358B to a lab bench.

3.2.3.3 Briefly spin down the previously-prepared lysates in a centrifuge, then add 2 µL of lysate to its corresponding PCR well or tube. Pipette up and down to mix.

3.2.3.4 Briefly centrifuge the PCR plate or tubes to concentrate reagents at the bottom of the tube.

3.2.3.5 Perform the reaction using the following set of cycling conditions:

Hot Start Touchdown PCR Conditions:

94°C	5 min	
94°C	30 sec	
AT*	30 sec	20 cycles at 55-45°, then
72°C	1 min	20 cycles at 45°C
72°C	7 min	
4°C	Hold	

* the first 20 cycles have an AT that decreases by 0.5°C for each cycle (touch down PCR). Then, 20 cycles with an AT at 45°C

3.2.3.6 Place the rack in the PCR machine, with a compression pad, and begin the cycle. Once the sample completes its first denaturation cycle (1min at 94°C) and the temperature begins to decrease, pause the program when it reaches 80°C, and wait for the temperature to stabilize.

3.2.3.7 Add 10µL of the Taq Mix to each PCR tube. Ensure all the tube caps are tightly closed, then close the thermocycler lid, wait 1 min, and resume the program.

3.2.3 Enzymatic Hot Start (AmpliTaq gold polymerase)

3.2.3.1 Dispense 48 µL of prepared master mix into one well per lysate in a 96-well PCR plate or into individual 0.2 mL PCR tubes. Cover the plate loosely or cover the tubes and hold on ice.

3.2.3.2 Remove the plate or tubes containing the master mix from room 358B to a lab bench.

3.2.3.3 Briefly spin down the previously-prepared lysates in a centrifuge, then add 2 µL of lysate to its corresponding PCR well or tube. Pipette up and down to mix.

3.2.3.4 Briefly centrifuge the PCR plate or tubes to concentrate reagents at the bottom of the tube.

3.2.3.5 Perform the reaction using the following set of cycling conditions:

Touchdown PCR Conditions:

94°C	10 min	
94°C	30 sec	
AT*	30 sec	20 cycles at 55-45°, then
72°C	1 min	20 cycles at 45°C
72°C	7 min	
4°C	Hold	

* the first 20 cycles have an AT that decreases by 0.5°C for each cycle (touch down PCR). Then, 20 cycles with an AT at 45°C

3.2.3.6 Place the rack in the PCR machine, with a compression pad, and begin the cycle.

3.2.3.7 Promptly remove the reactions from the thermocycler when complete. PCR product can be stored at 4°C for up to 1 week or at -20°C for up to 6 months.

3.3 Gel Electrophoresis Confirmation of Amplification

Refer to the "Gel Electrophoresis of PCR Product" SOP on the Food Safety Lab wiki.

3.4 ExoSAP Purification and Sequencing Submission

Refer to the "PCR Product Purification and Sanger Sequencing Submission" SOP on the Food Safety Lab wiki.

3.5 Sequence Analysis

Refer to the "Automated Sequence Editing" SOP on the Food Safety Lab wiki.

SECTION 4 TROUBLESHOOTING

4.1 **Issues with $MgCl_2$**

4.1.1 $MgCl_2$ forms a concentration gradient when frozen and needs to be vortexed prior to use.

4.1.2 Every PCR reaction has an optimal $MgCl_2$ concentration range, usually between 1-4 mM. Since Mg^{+2} forms complexes with dNTPs and acts as a cofactor for polymerase you may need to try several conditions to optimize your concentration.

4.2 **Too Much Enzyme**

4.2.1 Excess enzyme in your PCR can lead to smearing of PCR products.

4.3 **Wrong Primer Concentration**

4.3.1 If you have too little primer you won’t see any product.

4.3.2 Too much primer may result in primer dimerization, which may reduce amplification.

4.4 **Wrong PCR Program**

4.4.1 Check your program while it’s cycling to make sure it is the right program.

4.5 **Excess or Insufficient Template**

4.5.1 Too much template can inhibit PCR by binding all of the primers. If the lysate appears very turbid, dilute the template 1:10 or 1:100 and then use 1 µL of the diluted template for the PCR.

4.5.2 Too little template and amplification may be undetectable. Make sure you correctly prepared your dirty lysate and that you took a sufficiently large sample from your culture.

4.6 **Too Much dNTP or Degraded dNTP**

4.6.1 Excess dNTP inhibits PCR due to Mg2 depletion. Between 40-200 µM is optimal. dNTPs are sensitive to repeated freeze-thaw cycles. Make small aliquots when you get a fresh batch and turn over your stock frequently.

4.6.2 Note that the dNTP solution concentration suggested by the GoTaq Flexi manufacturer Promega suggests having each dNTP at 10mM , while this protocol uses 1mM due to less Mg2 being.

4.7 It may be wise to re-streak all cultures onto BHI plates if the organisms were isolated on LMPM and MOX plates. Make sure your positive control matches the plating medium of whatever you’re testing (i.e. if you’re testing cultures from LMPM plates, use a PC from an LMPM plate).

SECTION 5 REFERENCES